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14. ABSTRACT: Small molecule targeted at specific signal transduction pathways hold great promise for creating a new approach to prostate cancer treatment. However, resistance to these small molecule agents occurs through multiple mechanisms and restricts their therapeutic activity. Developing potential targets that can overcome this resistance by novel mechanisms is essential. In this proposal, the applicant research team demonstrates that resistance to small molecule AKT protein kinase inhibitors is potentially mediated by the Pim-1 protein kinase, and that unique Pim protein kinase inhibitors that can in turn synergize with AKT inhibitors to block prostate cancer growth overcome this resistance. The knowledge gained through the studies proposed in this application is essential for the development of this combined chemotherapeutic strategy.					
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DOD Technical Report

Introduction

In prostate cancer patients the PTEN phosphatase is absent in 50-80% of prostate cancer patients. When one combines this deletion with mutations in the PI3K and AKT itself it is likely that over 90% of prostate cancer patients have abnormalities in the AKT signaling pathway. Protein kinases have been widely targeted in an attempt to cure cancer and small molecules that regulate this pathway have entered the clinic. Unfortunately, preliminary laboratory studies suggest that inhibiting the AKT signal transduction pathway is not sufficient to inhibit prostate cancer growth. Investigators, including this team, have demonstrated that inhibiting the AKT pathway leads to increases in receptor tyrosine kinases that have the capacity to block the action of this kinase. In this proposal, the applicant research team demonstrates that resistance to small molecule AKT protein kinase inhibitors is potentially mediated by the Pim-1 protein kinase, and that unique Pim protein kinase inhibitors can in turn synergize with AKT inhibitors to block prostate cancer growth overcome this resistance. The knowledge gained through the studies proposed in this application is essential for the development of this combined chemotherapeutic strategy.

To carry out this research it is essential to be able to (1) understand the close interaction between AKT and Pim and (2) examine how Pim controls the level of cell surface receptor tyrosine kinases. This laboratory has in the past demonstrated that both Pim and AKT can regulate the phosphorylation of PRAS40, a protein that regulates the mTORC1 pathway. Additionally, both Pim and AKT modulate the phosphorylation of TSC-2 that also controls TORC1 activity. TORC1 plays a critical role in regulating 4E-BP1 phosphorylation, and thus modulates the activity of eIF4E and the translation of proteins that have a 5'Cap mRNA. Both of these results suggest that Pim can control protein translation and thus influence the growth of prostate cancer cells.

The focus of the research carried out in the first year of this grant has been to examine the ability of Pim to control protein translation. This focus builds on preliminary data collected and submitted at the time of this application.

Body

Experiments have been carried out to attack the tasks outlined for Year 1. These tasks are outlined and the importance of these findings discussed below each observation. A strategic decision was made to focus next on Task 3 from Year 2 of this grant followed by Task 1 of year 3. Although the order of analysis varies from the statement of work, these experiments have occurred in logical sequence and led *to an impressive and novel understanding of how Pim-1 regulates the level of receptor tyrosine kinases and thus the growth of prostate cancer. We have discovered that Pim-1 regulates the phosphorylation of eIF4B and being doing so is able to control the translation of the MET receptor tyrosine kinase in prostate cancer. This regulates the activity of the MET/HGF axis and potentially can affect the ability of these cells to spread and metastasize.*

Task 1, Year 1. Carry out experiments to examine whether inhibition of AKT with small molecule inhibitors increases the levels of Pim-1 mRNA? To determine the mechanism by which AKT inhibitors increase Pim-1 levels, PC3-LN4 cells were treated with either GSK690693 or MK2206 overnight and mRNA was isolated. As shown by qRT-PCR, both agents increase the levels of Pim-1 mRNA (**Fig 1a**). GSK690693 inhibits all three isoforms of AKT 1,2 and 3 while MK2206 inhibits only AKT 1,2. *These data suggest that inhibition of the AKT 1,2 isoform may be sufficient to increase Pim-1 levels*

transcriptionally. This further demonstrates that inhibition of AKT elevates Pim-1 through a transcriptional mechanism.

To attempt to replicate these findings derived from treating prostate cancer cells with small molecules, PC3-LN4 cells are treated with siRNAs either to AKT 1, 2 or AKT 1, 2, and 3. Results demonstrate that the level of Pim-1 mRNA increases with a decrease in AKT, but suggests that knocking down 1,2 and 3 was somewhat better than inhibiting the levels of 1,2 alone (**Fig. 1b**). Interestingly Western blots demonstrate that AKT-3 levels vary but are present in 5 prostate cell lines (**Fig. 1c**) but that they are significantly less than the level of AKT 1 and 2. *Taken together these results again validate the hypothesis that Pim-1 is regulated transcriptionally by AKT. Further experiments will need to be done to examine whether inhibition of AKT3 is needed for the control of Pim1 mRNA levels.*

Task 2, Year 1 - Determine whether FOXO transcription factors are capable of activating Pim-1 transcription.

To begin to evaluate the mechanism by which AKT inhibition activates the Pim-1 promoter, 3.0 kB of the Pim-1 upstream region was cloned into a firefly luciferase reporter plasmid and transfected into PC3-LN4 cells along with a renilla luciferase control plasmid. These cells were then treated with GSK690693 to block AKT and the ability of this agent to elevate Pim1 transcription evaluated. Results demonstrate that in a dose dependent fashion, AKT inhibition stimulates the activity of the Pim1 promoter (**Fig 2a**). *These results again demonstrate using a portion of the Pim1 promoter that inhibition of AKT stimulates the Pim1 transcription. These experiments set up a further analysis of which transcription factors might play a role in this stimulation.*

Because AKTi blocks the phosphorylation of the Foxo transcription factors, translocating these factors to the nucleus, the possibility that these proteins increased Pim1 transcription was next evaluated. qRT-PCR demonstrates that transduction of a cDNA containing an activated mutants of FOXO transcription factors increases Pim1 transcription 4-6 fold. *This result clearly points to a potential mechanism by which AKT inhibition activates Pim1 mRNA transcription (Fig. 2b). The experiment suggests that Foxo transcription factors play an important role here.*

To validate the further genetic manipulation of FOXO transcription factors is essential for the control of Pim1, siRNAs that target FOXO 1 or 3 or the combination of these proteins were transfected into prostate cancer and then GSK690693, the AKT inhibitor, applied to these cells. Results demonstrate that the decrease in the FOXO transcription factors blocked the induction of the transcription of Pim-1 by this AKT inhibitor (**Fig. 2c**). *Thus these results show by using two types of genetic manipulation that FOXO transcription factors are regulating Pim1 transcription. We suggest the following model. AKT inhibitors block the phosphorylation of FOXO transcription factors. This leads to their translocation to the nucleus and the binding to the Pim-1 promoter activating the transcription of the Pim-1 protein.*

Task 3, Year 1- Examine whether Pim-1 induced increases in receptor tyrosine kinases (RTKs) is cell line specific.

The addition GSK690693 an AKT inhibitor, induced up regulation of multiple RTK protein levels, including MET, EphA2, Her3, Her2, IGF1R, and the insulin receptor, in PC3-LN4 (**Fig. 3a**), and as shown MET, HER2, HER3 and EPHA2 in DU145, 22RV1, and VCAP cells (**Fig. 3b**). *These results demonstrate that the regulation of Pim-1 by AKT inhibition is a general phenomena that is not cell line specific.*

To document this further and demonstrate that Pim-1 plays an important regulatory role in the ability of AKT inhibitors to modulate RTKs, we determined the effects of Pim-directed siRNAs and small-molecule inhibitors on RTK levels. In all cell lines treated Pim-1 inhibitors blocked the ability of AKT inactivation to lead to increased RTK expression. Further, the use of siRNA directed at Pim-1 showed that a forced reduction in Pim-1 levels markedly reduced the ability of GSK690693 to elevate the protein levels of multiple RTKs, including MET and EPHA2, HER3, HER2, INSR, and IGF-IR, as well as the phosphorylation of ERK (**Fig. 3a**).

Thus, Pim1 is shown to play a role in the regulation AKT inhibitor regulation of RTK levels. The data obtained comes from experiments with both small molecules and genetic manipulation. This further establishes the role of Pim and the interaction with AKT.

Task 4, Year 1- Study the activity of Pim and AKT inhibitors on the growth of PC3-LN4 cells placed orthotopically in mice.

To evaluate the possibility that combining an AKT and Pim inhibitor would have dual activity in killing cancer cells Varying doses of GSK690693 plus SMI-4a were added to PC3-LN4 cells. Enhanced killing from this combination therapy was the result. (**Fig. 4a**). With this data, a Chou-Talalay combination index (CI) (32) was calculated using the Calcsyn from Biosoft and found to be < 0.5 . A CI less than 1.0 denotes a high degree of synergism between agents. When PC3-LN4 was grown in soft agar with a combination of both agents, but neither agent alone, tumor cell growth was inhibited (**Fig. 4b**). This combination therapy was then evaluated in an immunosuppressed mouse model by first injecting 10^{+6} PC3-LN4 cells subcutaneously. When tumors were palpable, animals were treated with GSK690693 30 mg/kg intraperitoneally for 7/7 days and SMI-4a 60 mg/kg orally twice a day for 5/7 days. The results demonstrate the ability of the combination, but not each agent alone, to inhibit tumor growth (**Fig. 4c**).

These novel biologic findings indicate that combination therapy aimed at inhibiting AKT and Pim kinases effectively blocks PCa growth, overcoming resistance mechanisms induced by AKTi therapy alone. This experiment also demonstrates that one can give combination therapy blocking both of these kinase pathways without harming the animals. As better inhibitors of both pathways are developed this combined therapy should become more powerful. Further experiments are carried out and described below which focus on a single receptor, MET, and how this receptor may be modulated biochemically by Pim.

Task 3 –Year 2. Using IHC, examine whether in normal mice increases in Pim-1 lead to increases in receptor tyrosine kinases (RTKs).

Our preliminary results suggested that the Pim protein kinase could regulate multiple RTKs. However, it was not possible to analyze multiple proteins simultaneously. Because the MET tyrosine kinase drives the growth and metastasis of multiple human cancer types (1, 2), we first examined whether the levels of Pim-1 correlates with the level of MET in prostate tumor cells. Immunohistochemical analysis of a high-density tissue microarray representing tissues from 27 patients with newly diagnosed prostate cancer indicated that the levels of Pim-1 and MET are highly correlated [correlation coefficient (R) = 0.85 (**Fig. 5a**).

To determine whether Pim-1 expression has a direct effect on MET protein levels, we used several different approaches. RNA interference-mediated silencing of Pim-1 expression markedly reduced both the total and cell surface (**Fig. 5b**) expression of MET in the prostate tumor cell line PC3-LN4 (3). Moreover, although the overexpression of wild-type Pim-1 in DU145 prostate cancer cells resulted in

increased levels of MET protein, the overexpression of kinase-dead Pim-1 did not (**Fig. 5c**). The phosphorylation of MET and AKT that occurs on culture of wild-type DU145 cells in the presence of HGF was enhanced in the Pim-1-overexpressing cells (**Fig 6a**). This effect was specific as there was no difference in ERK phosphorylation between the over expressor and wild-type cell lines cultured in HGF. Conversely, in PC3-LN4 cells in which Pim levels were reduced using siRNA, the HGF-induced phosphorylation of MET and AKT was lower than those in control cells (**Fig. 6b**).

Potentially, Pim-1 could regulate MET protein levels without having a significant physiologically relevant effect on the response on the cells to HGF. HGF induces scattering of DU145 cells (4). Culture of the Pim-1-overexpressing DU145 cells with HGF resulted in a pattern of scattering of the cells similar to that observed in HGF-treated wild-type DU145 cells; however, scattering was observed at a lower concentration of HGF (0.25 ng/ml vs. 1 ng/ml) (**Fig. 6c**). Pretreatment with a MET inhibitor (PHA665752) blocked the HGF-induced cell scattering in the Pim-1 overexpressing cells (**Fig. 6d**) suggesting that this Pim-1 effect is mediated through the MET receptor. Taken together, these data suggest that the Pim-1-induced increase in the MET receptor enhances the ability of HGF to signal in these tumor cells.

Finally, to evaluate the ability of Pim-1 to modulate HGF-induced invasion of tumor cells, PC3-LN4 cells were placed in the upper chamber of a Boyden chamber in which the membrane had been coated with Matrigel, and HGF was added to the media in the lower chamber. In this assay, the Pim-1-overexpressing PC3-LN4 cells demonstrated increased invasion through the Matrigel-coated membrane, and this invasion was blocked by treatment with the MET inhibitor PHA665752 (**Fig. 6e**) or knockdown of Pim-1 expression by siRNA (**Fig. 6f**).

Collectively, these results demonstrate that Pim-1 levels are correlated to the MET RTK in prostate cancer, and Pim-1 can control signaling through the HGF/MET axis and that this affects HGF-induced cell motility and invasion tumor cells. *The impact of these findings are such that they imply that if one regulates Pim-1 activity with small molecules drugs, it may be possible to modulate prostate tumor invasion and metastasis.*

Task 1. Year 3 - Examine whether Pim-1 kinases regulate the levels of RTKs by controlling transcription, protein translation, or the stability of these receptors.

Identification of the mechanisms by which Pim-1 controls the level of expression of MET requires a more detailed understanding of the mechanisms underlying translation of the MET protein. The 5'UTR of MET is relatively long (408 nt) and GC-rich, which is consistent with the possibility that this region functions as an internal ribosome entry site (IRES). Modeling of the secondary structure using the MFOLD program (5) indicated that the 5'UTR of MET is highly structured with the overall folding energy of the most stable predicted structure being - 293.3 kcal/mol.

The translation initiation factor, eIF4B preferentially promotes the translation of mRNAs containing such complex secondary structures in their 5'UTRs (6). Notably, eIF4B has been identified as a potential substrate of Pim-1 kinase activity (7, 8). Using *in vitro* kinase assays in which FLAG-tagged eIF4B was expressed in 293T cells and immunoprecipitated with anti-FLAG antibody, we confirmed that Pim-1 phosphorylated wild-type eIF4B. To identify the phosphorylation site(s), we transfected the cells with eIF4B constructs with S406A and S422A mutations, which prevent phosphorylation at these sites. Pim kinase was capable of phosphorylating an eIF4B construct with an S422A mutation, but not eIF4B with an S406A mutation (**Fig. 7a**). Similar results were obtained using commercially available recombinant eIF4B with Pim-1 phosphorylating eIF4B at S406 and to a lesser extent at S422 (**Fig. 7b**).

Phosphorylation at both these sites was inhibited by the small molecule pan-Pim kinase inhibitor GNE652 (produced by Genentech) (**Fig. 7b**). In marked contrast, in agreement with a previous report (9), we found that AKT1 preferentially phosphorylated eIF4B at S422 and confirmed that this phosphorylation was inhibited by GSK690693, a small molecule AKT inhibitor (**Fig. 7b**).

To determine whether the Pim kinase phosphorylation of eIF4B occurs in tumor cells, we analyzed Pim siRNA-transfected PC3-LN4 (**Fig. 7c**) cells. The knockdown of Pim-1 expression was associated with reduced phosphorylation of eIF4B S406 but did not affect phosphorylation on S422. Insulin and serum-stimulation of serum-starved control tumor cells resulted in enhanced eIF4B phosphorylation at S406 and this effect was impaired in the Pim siRNA-transfected PC3-LN4 (**Fig. 7c**) cells. Insulin and serum-stimulation of serum-starved control tumor cells also resulted in enhanced eIF4B phosphorylation at S422. In siRNA-transfected tumor cells a decrease in Pim levels impaired the insulin-treated S422 phosphorylation, but it did not affect the serum-stimulated levels (**Fig. 7c, 7d**). Interestingly, both insulin and serum increased the expression of Pim-1 protein (**Fig. 7c, 7d**), suggesting another level of regulation of eIF4B phosphorylation under these conditions. In these cells the phosphorylation of S406 and, to a lesser extent, S422 was blocked by the Pim inhibitor GNE652 (**Fig. 7e**). Moreover, treatment of PC3-LN4 and DU145, with either of two pan-Pim inhibitors, GNE652 or AZD1208, reduced the phosphorylation of eIF4B S406.

Together these data suggest that S406 is the major Pim directed phosphorylation site on eIF4B, while S422 phosphorylation could occur as a result of interacting signaling pathways driven by specific growth factors in individual cell lines. As suggested in the introduction to this year 1 update it would appear that AKT and Pim are working together to regulate translation with Pim-1 phosphorylating the S406 site on eIF4B and AKT targeting S422.

Importantly, these results point to eIF4B as a biomarker for both Pim-1 activity that can potentially be used to modulate the use of Pim-1 inhibitors.

It has been suggested that insulin-induced phosphorylation of eIF4B S406 is dependent on both MEK and mTOR activity (9). As described above, we found that treatment of PC3-LN4, and DU145, with GNE652 or AZD1208 (two Pim-1 inhibitors produced by Genentech and AstraZeneca) markedly reduced the phosphorylation of eIF4B S406. In contrast, treatment of these cell lines with the PI3K/AKT/mTOR pathway inhibitors BEZ235, PP242, or U0126 blocked the phosphorylation of eIF4B S422 (**Fig. 8a**) and had no effect on S406. As the results could be affected by the experimental conditions, we analyzed eIF4B phosphorylation in PC3-LN4 that were either grown in serum-rich conditions (**Fig. 8a**) or starved and insulin-stimulated (**Fig. 8b**). Again, we found that phosphorylation of eIF4B S406 was not inhibited by small molecules that block the PI3K/AKT/mTOR pathway (MK2206, rapamycin, PP242, and BEZ235) or the MEK pathway (U0126, and BI-D1870), when used alone or in combination. In marked contrast, the Pim inhibitors, GNE652 and AZD1208, clearly blocked phosphorylation of eIF4B S406. These results suggest that phosphorylation of eIF4B S422 is predominantly controlled by the PI3K/AKT/mTOR pathway, with MEK also playing a role in phosphorylation of S422 whereas phosphorylation of eIF4B S406 is predominantly controlled by the Pim protein kinases.

We have found previously that small molecule AKT inhibitors can induce Pim-1 expression. Consistent with this observation, GSK690693, an AKT inhibitor, increased the phosphorylation of eIF4B S406 while reducing the levels of eIF4B S422 phosphorylation (**Fig. 8c**). This AKT inhibitor-induced increase in phosphorylation of eIF4B S406 was blocked by GNE652, a Pim inhibitor, suggesting that under these circumstances the increased Pim-1 expression associated with inhibition of the AKT protein kinase plays a role in the phosphorylation of eIF4B S406.

These results clearly demonstrate that inhibitors of Pim-1 that are entering clinical trials can regulate the phosphorylation of the important translational control factor, eIF4B. Other inhibitors do not regulate the phosphorylation of this protein on S406 suggesting that Pim-1 inhibitors will play an important, if not essential, role in regulating translation in tumor cells.

The protein translation initiation complex consists of several proteins in the eIF family. In yeast and mammalian cells (9, 11), the binding of eIF4B to eIF3, a 13 member complex, enhances protein synthesis. It has been suggested that phosphorylation of eIF4B-S422 can regulate its interaction with the eIF3 (12). To examine whether the association of eIF3 proteins with eIF4B is affected by eIF4B-S406 phosphorylation, we carried out co-immunoprecipitation experiments using cells transfected with the phosphorylation site S406A and S422A mutant constructs as well as S406D and S406E mutant constructs, which carry negative charges and thus mimic phosphorylation of eIF4B at S406. 293T cells were first transfected with wild-type FLAG-eIF4B or the phosphorylation site mutant constructs, starved of serum overnight, and then stimulated with insulin to promote the formation of the eIF3 complex. Consistent with the reported effects, insulin treatment stimulated an enhanced interaction of wild-type eIF4B with eIF3A and eIF3B and this interaction with eIF3A was absent when the cells were transfected with the S422A or S406A/S422A mutants (**Fig. 9a**). This interaction also was abrogated when the cells were transfected with the S406A construct; moreover, the S406D and S406E mutant constructs, mimicking the Pim phosphorylation site, formed a complex with eIF3A or eIF3B as efficiently as wild-type eIF4B (**Fig. 9b**). Pretreatment of 293T cells with the pan-Pim inhibitor GNE652 or the PI3K/mTOR blocker BEZ235, or both agents prior to insulin addition blocked the ability of eIF4B to bind to eIF3B (**Fig. 9c**). Thus, eIF4B S406 phosphorylation plays an essential role in the formation of the translation initiation complex.

These data suggested that both the eIF4B S406 and S422 phosphorylation sites contribute to the binding of eIF4B to eIF3B and that inhibition of phosphorylation of both sites resulted in greater inhibition of binding than inhibition of either site alone. We therefore extended the studies to determine whether the phosphorylation state of eIF4B affects the formation of translation initiation complex at the 5' cap structure. The m7-GTP-sepharose binding assay was used to identify the proteins that bound to the 5' cap structure. Insulin treatment of starved PC3-LN4 cells enhanced the binding of both eIF4B and eIF3B to the m7-GTP beads (**Fig. 9d**). Treatment with the Pim inhibitor GNE652 reduced binding of both eIF4B and eIF3B to the m7-GTP beads as did treatment with the PI3K/mTOR pathway inhibitor, BEZ235 (**Fig. 9d**). Concomitant treatment with both GNE652 and BEZ235 resulted in the same or better reduction in binding. The inhibitors did differ, however, in terms of their effects on the binding of other components of the translation complex; BEZ235 enhanced the interaction of 4EBP1 with the complex, but GNE652 did not. Consistent with this observation, BEZ235 treatment also resulted in a decrease in the binding of eIF4G, whereas GNE652 did not (**Fig. 9d**). These findings suggest eIF4B S406 phosphorylation is an important regulatory element in controlling the protein binding of eIF4B to the eIF3 complex and that eIF4B S406 and S422 phosphorylation may play different roles in the formation of the translation initiation complex.

The conclusions from this data demonstrate the biologic importance of eIF4B phosphorylation by Pim-1. They suggest that this phosphorylation of this protein is key in regulating translation through binding to the eIF3 complex.

The experiments described above established that Pim-1 kinase activity may affect translational activity directly. Furthermore, we have previously demonstrated that Pim-1 does not affect the transcription of MET (3). To specifically determine the role of eIF4B S406 phosphorylation in the regulation of the

translation of MET, we utilized the eIF4B mutant constructs. Transfection with the wild-type eIF4B construct increased the expression of MET as compared to the vector control (**Fig. 10a**) whereas transfection with the S406A and S406A/S422A mutants failed to do so. The levels of MET protein were increased to the levels seen on transfection with wild-type eIF4B after transfection with eIF4B-S422A (**Fig. 10a**), S406D, or S406E (**Fig. 10b**) mutants. Furthermore, treatment with the small molecule Pim inhibitor, AZD1208, caused down regulation of MET expression that was reversed by transfection with wild-type eIF4B, eIF4B S406D or S406E, but not by eIF4B S406A (**Fig. 10c**). Thus, the eIF4B S406 phosphorylation site is critical for the regulation of MET translation in this cell line.

To determine the effects of eIF4B S406 phosphorylation on translation of MET protein, we monitored new protein synthesis rates by labeling PC3-LN4 cells with [³⁵S]-methionine. Treatment of both PC3-LN-4 with the pan-Pim inhibitor AZD1208, but not the PI3K/mTOR inhibitor BEZ235, reduced MET expression, but had no effect on ERK protein synthesis. While in comparison, cycloheximide treatment decreased the levels of both MET and ERK (data not shown). In the same cell system, overexpression of wild-type eIF4B, S406D and S406E mutants, but not the S406A protein, increased the rate of MET protein synthesis (**Fig. 10d and 10e**).

These experiments demonstrate that eIF4B phosphorylation by Pim-1 plays a significant role in regulating eIF4B phosphorylation. The phosphorylation of this translation factor then controls the production of MET protein while having no effect on the levels of the ERK serine threonine protein kinase. In comparison, cycloheximide that is a general translation regulator controls the expression of both of these proteins.

In summary, importantly we have discovered a new substrate for the Pim-1 protein kinase eIF4B that is essential for controlling the translation of the MET protein. Inhibiting this phosphorylation in prostate cancer cells decreases MET levels and consequently diminishes the activity of the MET/HGF axis with diminished invasion and motility of these tumor cells. AKT is shown to share yet another substrate with Pim and thus enhance our hypothesis that inactivating both of these pathways will be needed to block prostate cancer growth.

Key Research Accomplishments:

- 1- Inhibition of Pim by RNA interference or pharmacologic inhibition blocks AKT inhibitor-induced upregulation of RTKs in prostate cancer cells.
- 2- Small molecule AKT inhibitors increase the levels of Pim-1 in multiple cell lines.
- 3- Foxo transcription factors are capable of regulating Pim-1 transcription.
- 4- Pim-1 induced increases in receptor tyrosine kinases are not cell line specific.
- 5- The expression of RTKs is controlled by Pim-1 in a cap-independent manner by controlling internal ribosome entry.
- 6- Combination of Pim and AKT inhibitors resulted in synergistic inhibition of prostate cancer growth.
- 7- Pim protein levels correlate with MET levels in prostate cancer
- 8- Pim controls the phosphorylation of eIF4B
- 9- eIF4B phosphorylation regulates the association of this protein with the eIF3 complex
- 10- Pim-directed eIF4B phosphorylation modulates the translation and activity of MET

Conclusion:

The PI3K/AKT pathway is hyperactivated in prostate cancer but its effective therapeutic targeting has proven difficult. In particular, the antitumor activity of AKT inhibitors is attenuated by upregulation of receptor tyrosine kinases (RTK) through an uncharacterized feedback mechanism. In this report, we show that RNA interference-mediated silencing or pharmacologic inhibition of Pim-1 activity curtails AKT inhibitor-induced upregulation of RTKs in prostate cancer cells. Although Pim kinases have been implicated in cap-dependent translational control, we find that in the context of AKT inhibition, the expression of RTKs is controlled by Pim-1 in a cap-independent manner by controlling internal ribosome entry. Combination of Pim and AKT inhibitors resulted in synergistic inhibition of prostate tumor growth in vitro and in vivo.

Focusing on a specific receptor tyrosine kinase MET we find that Pim through regulation of eIF4B phosphorylation is able to control the synthesis of this protein and thus modulate the sensitivity to the HGF/MET axis in prostate cancer.

The next potential test will be to examine whether Pim inhibitors alone or in combination with AKT block prostate cancer metastasis in animals, and inhibit the growth of this cancer when combined with AKT inhibitors. In addition to MET it would of importance to examine more broadly the activity of Pim inhibitors on other RTKs that drive the growth of prostate cancer.

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Supporting data:
Figures 1 – 10

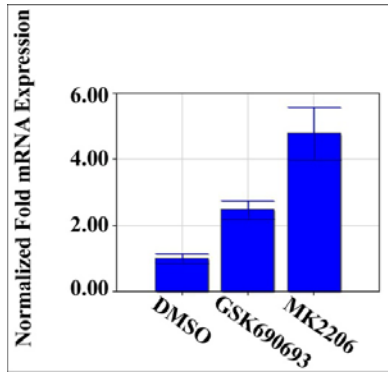


Figure 1a: PIM-1 mRNA levels are stimulated by AKT inhibitors. PC3-LN4 were treated with GSK690693 or MK2206 (10 μ M) for 18h and mRNA isolated. qRT-PCR was done using β -actin as a control. The S.D. of triplicate experiments is shown.

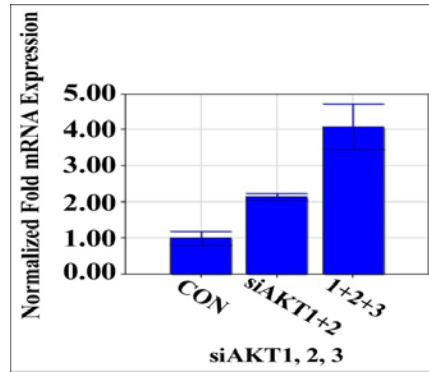


Figure 1b: PIM-1mRNA is increased by knock-down of AKT with siRNA. PC3-LN4 cells were treated with scrambled siRNA (con) or siRNA to AKT 1,2 or AKT 1,2 and 3. The experiment was done in triplicate. 48h after transfection mRNA was isolated and qRT-PCR was done using β -actin as a control. The S.D. of these determinations is shown.

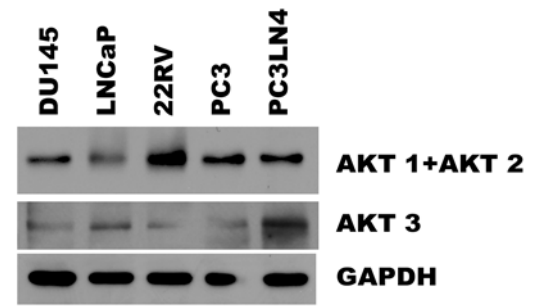


Figure 1c: Whole cell lysates were probed after Western blotting with an Ab to AKT 1 plus AKT 2 and AKT 3.

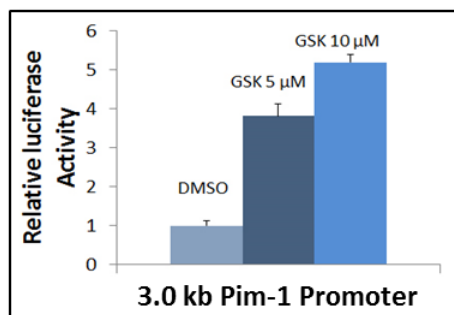


Figure 2a: GSK690693 stimulates the Pim-1 promoter. 3.0 k b of the Pim-1 promoter was cloned into a luciferase vector and transfected into PC3-LN4 cells which were then treated with GSK690693. The experiment was repeated in triplicate and the S.D. from the mean is shown.

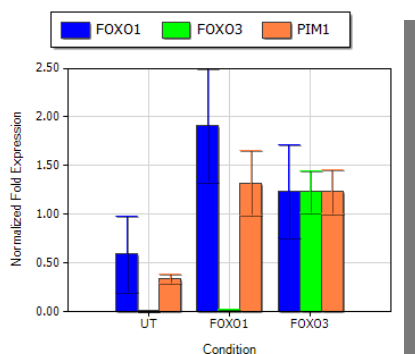


Figure 2b: Expression of FOXO1 (AAA) and FOXO3a (AAA) increases **PIM1 mRNA levels**. **PC3LN4** cells were transfected with empty vector, Myc-FOXO1 (T24A, T253A, S316A) or Flag-FOXO3a (T32A, S253A, S315A) plasmids, and 48 h later PIM1 mRNA levels were determined by QT-PCR using b-Actin as the reference gene. Values are the average of 3 measurements $\pm$ S.D. mean.

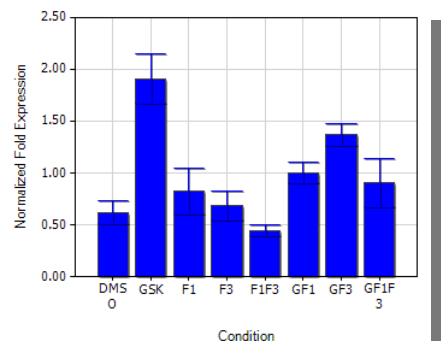


Figure 2c: Knock-Down of FOXO1 and FOXO3 decreases PIM1 mRNA levels: PC3-LN4 cells were treated with DMSO, GSK (5 μ M) or transfected with FOXO1 (F1) and FOXO3 (F3) siRNAs either alone or in combination with GSK treatment (GF1, GF3, GF1,3) for 72 h. Total RNA was isolated and relative amounts of PIM1 mRNA were determined by qRT-PCR and normalized to Actin mRNA. Independent replicate experiments yielded similar results and the $\pm$ S.D for 6 determinations is shown.

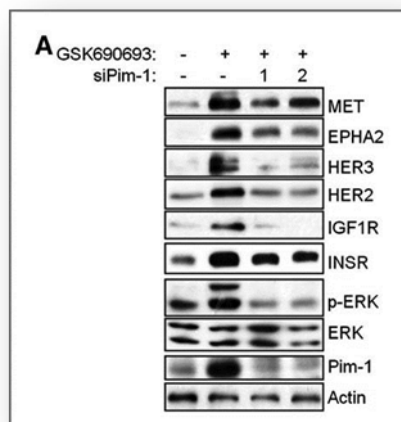


Figure 3a: Pim-1 is required for elevated expression of RTKs induced by AKT inhibition. Immunoblot analyses were carried out with the indicated antibodies in PC3-LN4 cells that were treated with 2 different siRNAs (1 and 2) against Pim-1 as well as a nontargeting control siRNA (2 left lanes) for 48 hours followed by the addition of GSK690693 (5 μ mol/L) for an additional 24 hours.

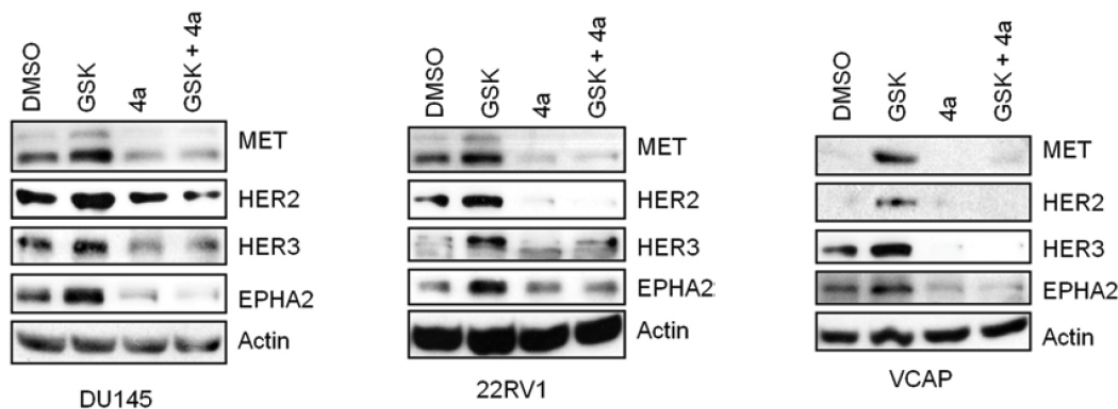


Figure 3b: GSK690693 induced RTK up-regulation is blocked by a Pim kinase inhibitor in prostate cancer cells suggesting that Pim regulates RTK elevation in multiple cell lines. Prostate cancer cell lines DU145, 22RV1, and VCAP were treated with GSK690693 (GSK 5 μ M) or SMI4a, 10 μ M) or the combination of both compounds for 24h. Whole cell lysates were subjected to immunoblot analyses with the indicated antibodies. Data are representative of at least two independent experiments.

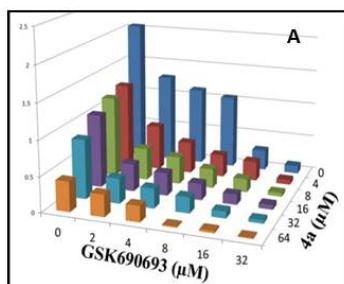


Figure 4a: Combination treatment of PC3-LN4 cells with GSK690693 and SMI-4a synergistically kills PSA cells. Cells were treated with the indicated doses of compounds for 48h and an MTS assay was done in triplicate.

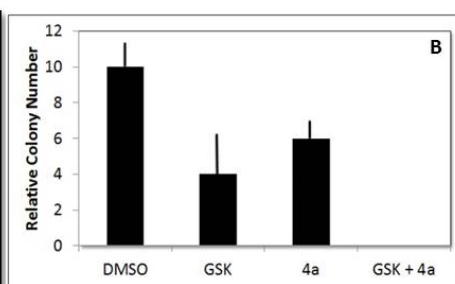


Figure 4b: Dual treatment with GSK690693 and SMI-4a inhibits soft agar growth of PC3-LN4 cells. PC3-LN4 were plated 5×10^3 per 35 mm dish and treated with GSK690693, SMI-4a or the combination. 100 fields were counted and the mean number of colonies/field $\pm$ S.D. is shown.

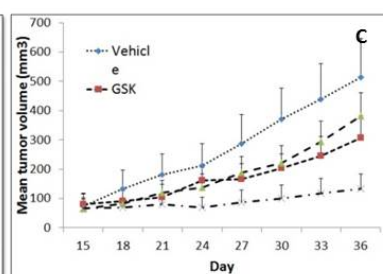


Figure 4c: Combination therapy inhibits growth of PC3-LN4 tumors grown subcutaneously in immunocompromised mice. 40 NOD/SCID mice were injected with 1×10^6 PC3-LN4 cells. When tumors were visible on day 15 in groups of 10 they were treated with vehicle, either agent alone or the combination. The experiment was terminated on day 36. The mean size of the tumor $\pm$ S.D. is shown.

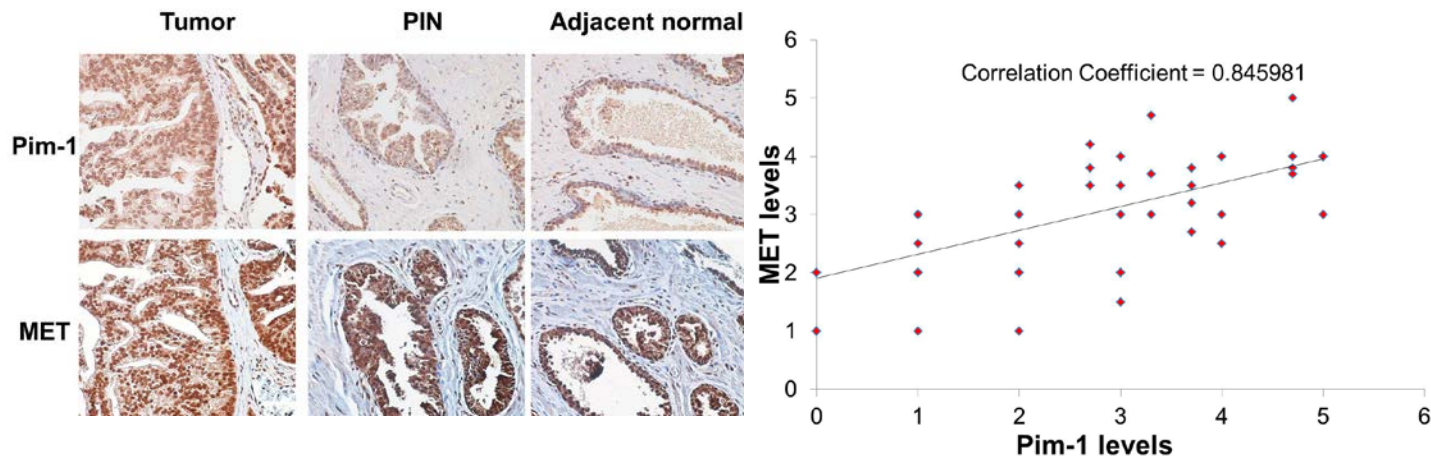


Fig. 5a

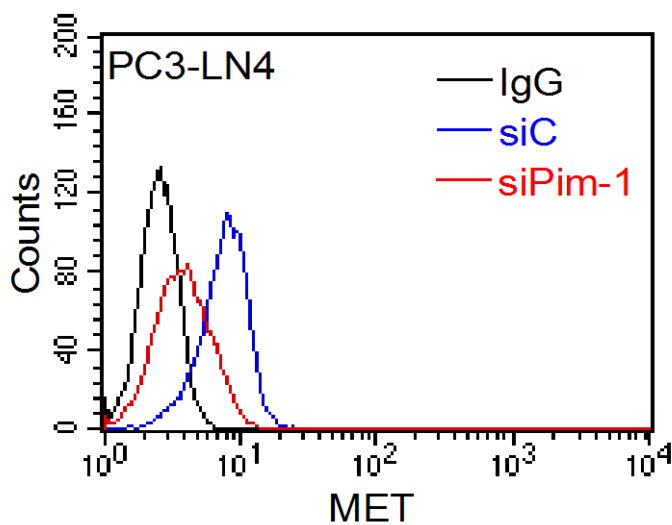


Fig. 5b

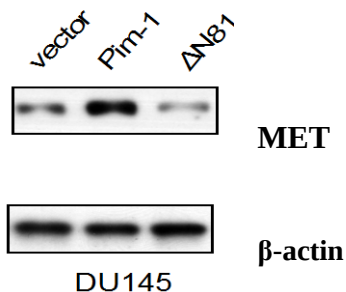


Fig. 5c

Fig. 5. The Pim-1 kinase regulates MET expression. (A) Representative images of a human prostate tissue microarray stained with anti-MET and anti-Pim-1 antibodies against normal, prostatic intraepithelial neoplasia (PIN), and tumor tissue. The relative strength of antibody staining was plotted as Pim-1 vs MET. The correlation coefficient (R) was derived by Microsoft Excel analysis. (B) PC3-LN4 were treated with siRNA targeting Pim-1 (siPim-1) or a non-targeting control (siC) for 72 hours. The level of cell surface MET expression was visualized by flow cytometry using anti-MET antibody and R-Phycoerythrin (PE)-conjugated secondary antibody. (C) DU145 cells were transfected with Pim-1 or its kinase dead mutants Δ N81 expressing plasmids. After 48 hours and the levels of Pim-1, MET and the loading control β -actin were examined by Western blotting.

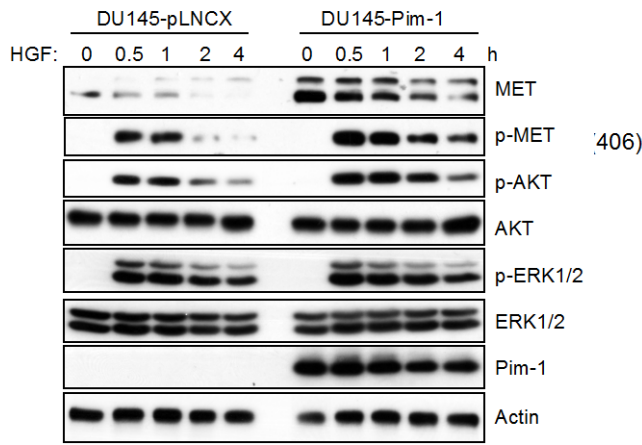


Fig. 6a

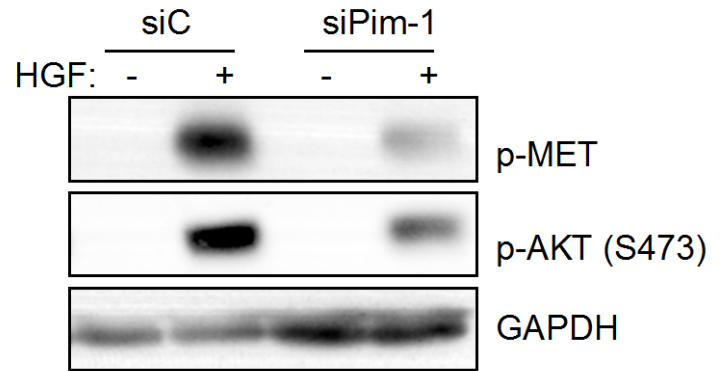


Fig. 6b

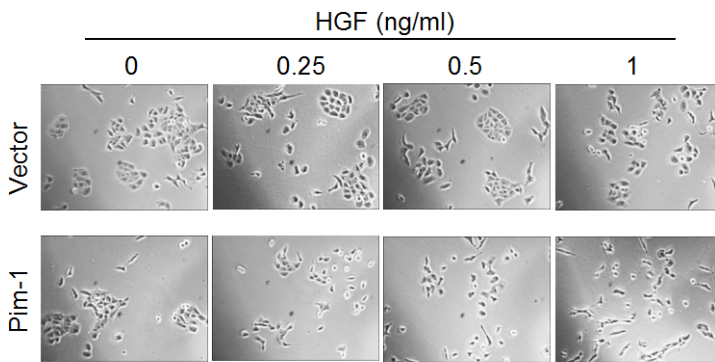


Fig. 6c

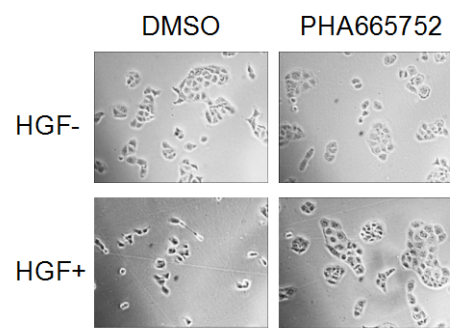


Fig. 6d

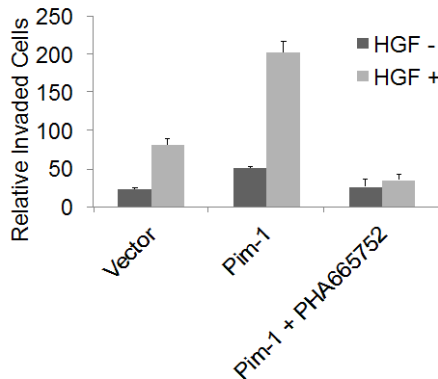


Fig. 6e Fig. 6f

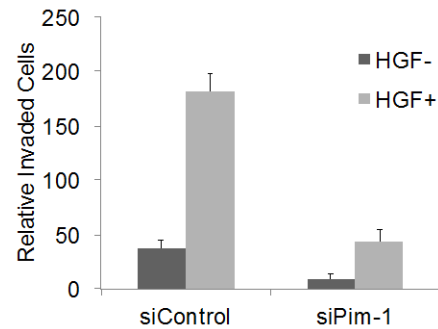


Fig. 6. Pim-1 regulates HGF-MET signaling and cell motility. **(A)** DU145 cells expressing an empty vector or Pim-1 were serum-starved for 24 hours before treated with 100 ng/ml HGF for indicated times. Cell lysates were analyzed by immunoblot assays using the indicated antibodies. **(B)** PC3-LN4 cells were transfected with Pim-1 siRNA or a control siRNA for 48 hours. Cells were serum-starved for 24 hours before treated with 100 ng/ml HGF for 30 minutes. Cell lysates were analyzed by immunoblot assays using the indicated antibodies. **(C)** DU145 cells expressing an empty vector or Pim-1 were treated with increasing concentrations of HGF for 24 hours. **(D)** DU145 cells expressing Pim-1 were treated with 0.25 ng/ml of HGF for 24 hours in the presence of PHA665752 (1 μ mol/L) or DMSO. Microphotographs show cell scattering effect. Scale bar, 100 μ M. **(E, F)** The invasion of PC3-LN4 cells was assayed using a Boyden chamber with the membrane coated with Matrigel. HGF (100 ng/ml) was added to the lower chamber and in specific experiments PHA665752 (1 μ mol/L) was added to the upper chamber. Cells were transfected with a Pim-1 expressing plasmid **(E)** or Pim-1 siRNA **(F)** for 48 hours prior to HGF addition. The average $\pm$ S.D. is shown.

Fig. 7a

Fig. 7b

Fig. 7c

Fig. 7d

Fig. 7e

Fig. 7. Pim-1 directly phosphorylates eIF4B Ser406. **(A)** FLAG-tagged eIF4B as well as its mutants were expressed in cells and immunoprecipitated using anti-FLAG antibody with Protein A/G-Sepharose beads. The beads were incubated with 100 ng of purified Pim-1 proteins at 37 °C for 30 minutes. Samples were analyzed by immunoblot assays using indicated antibodies. **(B)** Pim-1 controls eIF4B phosphorylation at Ser406. Purified eIF4B and Pim-1 or AKT1 were incubated in the presence or absence of GNE652 (0.1 μmol/L) or GSK690693 (0.1 μmol/L). Samples were subjected to immunoblotting analysis with the indicated antibodies **(C)** Cells were treated with Pim-1 siRNA for 48 hours, then cells were serum-starved for 24 hours before stimulated with insulin (1 μg/ml) or 20% FBS for an additional 30 minutes. Cell lysates were analyzed by immunoblot assays using indicated antibodies. **(D)** PC3-LN4 cells were treated with Pim-1 siRNA for 48 hours, then cells were serum-starved for 24 hours before stimulated with insulin (1 μg/ml) or 20% FBS for additional 30 minutes. Cell lysates were analyzed by immunoblot assays using indicated antibodies. **(E)** Pim-1 dependent regulation of eIF4B Ser406 phosphorylation in response to growth factors and during the cell cycle. Cells were serum-starved for 24 hours and pretreated with GNE652 (1 μmol/L) for 3 hours before stimulation with insulin (1 μg/ml), HGF (100 ng/ml), or EGF (100 ng/ml) for 30 minutes. Cell lysates were analyzed by immunoblot assays using the indicated antibodies.

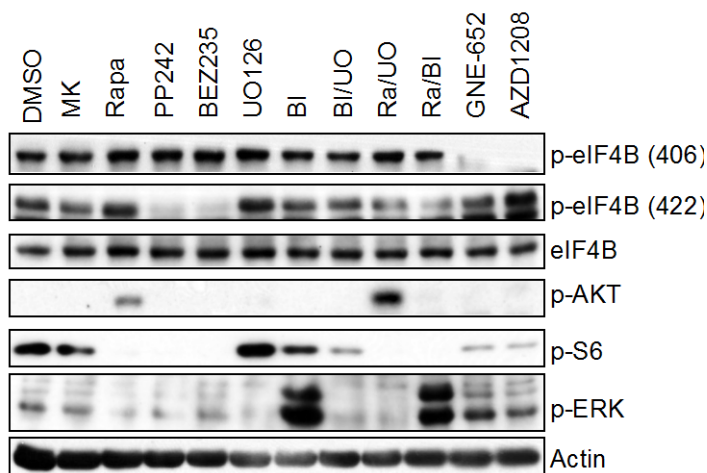


Fig. 8a

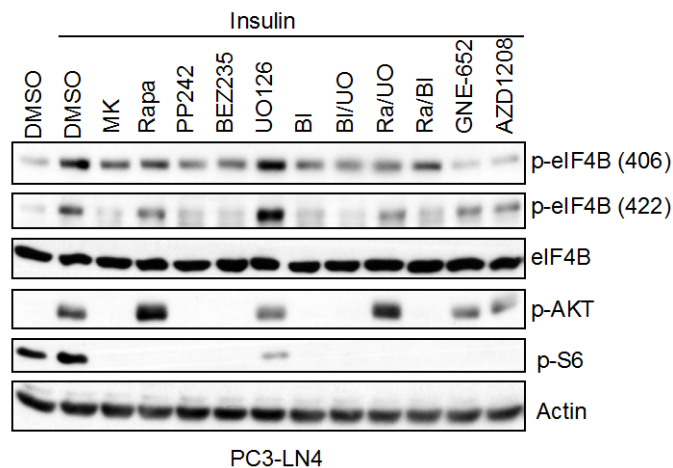


Fig. 8b

GSK690693 : 0 0.625 1.25 2.5 5 5 (μmol/L)
 GNE-652 : 1 1 (μmol/L)

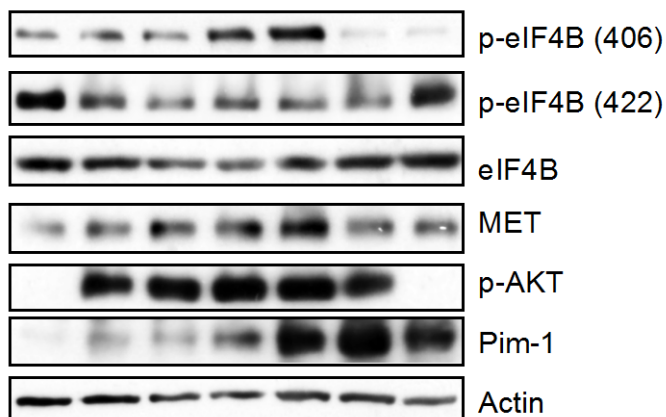


Fig. 8c

Fig. 8. Pim and PI3K/AKT/mTOR pathways control phosphorylation of eIF4B Ser406 and Ser422, respectively. **(A)** Cells were treated with MK2206 (1 μmol/L), Rapamycin (Rapa, 100 n mol/L), PP242 (1 μmol/L), BEZ235 (0.5 μmol/L), UO126 (UO, 15 μmol/L), BI-D1870 (BI, 10 μmol/L), GNE652 (1 μmol/L), AZD1208 (3 μmol/L), and the indicated inhibitor combination for 3 hours. Cell lysates were analyzed by immunoblot assays using indicated antibodies. **(B)** PC3-LN4 cells were serum-starved for 24 hours and pretreated with inhibitors before stimulating with insulin. **(C)** PC3-LN4 cells were treated with GSK690693 and GNE652 as indicated for 24 hours. Cell lysates were analyzed by immunoblot assays using the indicated antibodies.

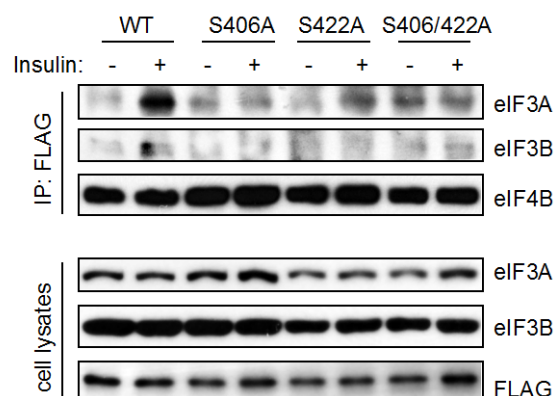


Fig.9a

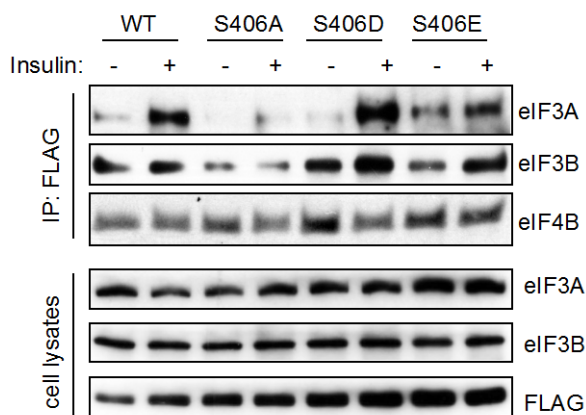


Fig. 9b

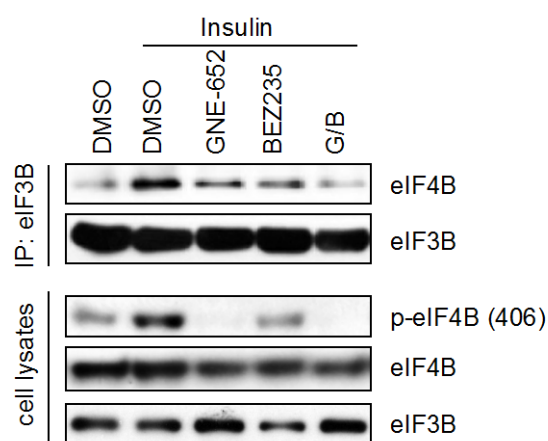


Fig. 9c

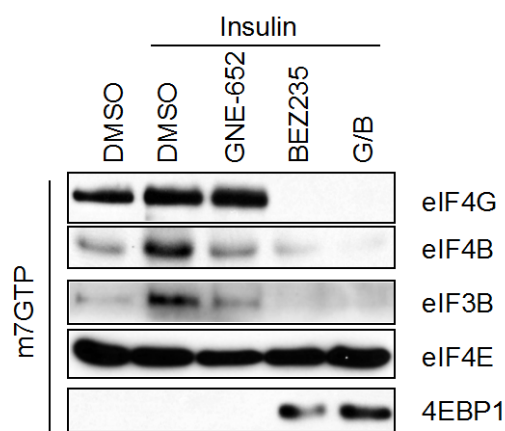


Fig. 9d

Fig. 9. Phosphorylation of eIF4B regulates its association with the eIF3 translational complex. **(A, B)** FLAG-tagged wild-type (WT) eIF4B and its mutants were expressed in cells. Cells were stimulated with insulin (1 μ g/ml) for 15 minutes. Anti-FLAG antibodies were used to immunoprecipitate the translation complex. Immunoprecipitates were Western blotted with antibodies as shown. Cell lysates used in these immunoprecipitations were immunoblotted as shown. **(C)** Cells were serum-starved and pretreated with GNE652 (1 μ mol/L), BEZ235 (0.5 μ mol/L), or the combination before stimulated with insulin (1 μ g/ml) for 15 minutes. Co-immunoprecipitation was carried out with anti-eIF3B antibody and the immunoprecipitates Western blotted for eIF4B and eIF3B. Lysates used for immunoprecipitation were immunoblotted as labeled **(D)** Cells were treated as in **(C)**. Cell lysates were incubated with m7-GTP-Sepharose as per manufacturer's instructions and these beads were analyzed by immunoblot assays using indicated antibodies.

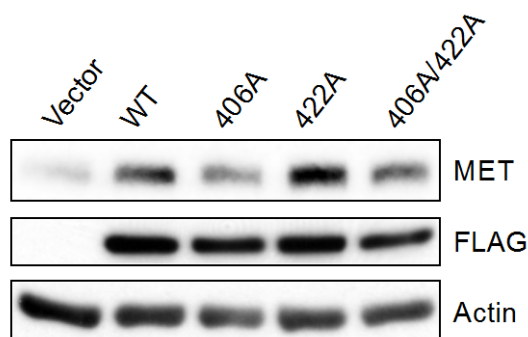


Fig. 10a

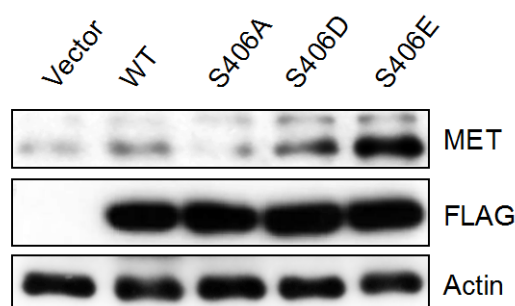


Fig. 10b

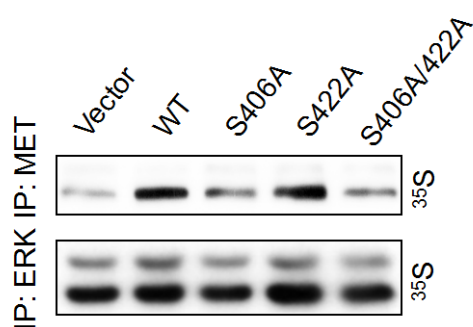


Fig. 10c

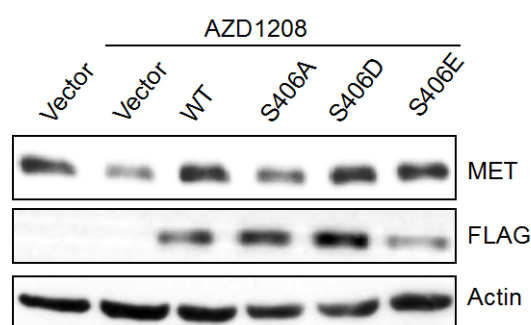


Fig. 10d

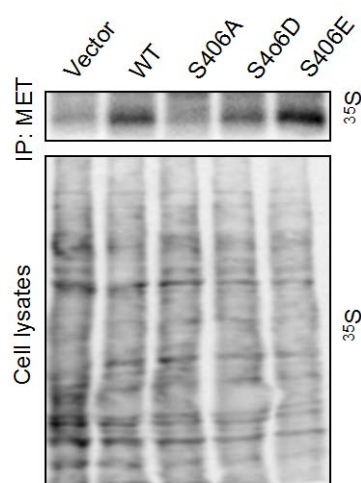


Fig. 10e

Fig.10. eIF4B Ser406 phosphorylation positively regulates the MET expression. (A, B) Cells were transfected with pcDNA3 (Vector), or plasmids expressing wild-type eIF4B (WT) and its mutants for 48 hours. Cell lysates were analyzed by immunoblot assays using indicated antibodies. (C) Cells were transfected as in A and B. 24 hours after transfection cells were treated with AZD1208 (3 μ mol/L) for additional 24 hours. Cell lysates were analyzed by immunoblot assays using indicated antibodies. (D, E) Cells overexpressing eIF4B or its mutants S406A, S422A, S406/422A, S406D, and S406E were labeled with 35 S for new protein synthesis. Newly synthesized MET was immunoprecipitated and separated by SDS-AGE, and visualized by autoradiography.